High-dose methotrexate ameliorates collagen-induced arthritis but does not inhibit the release of proinflammatory cytokines by peritoneal macrophages in mice

BERNADETA NOWAK[1], PIOTR GŁUSZKO[2], MARTA CISZEK-LENDA[1], MAŁGORZATA ŚRÓTTEK[1], BEATA KWAŚNY-KROCHIN[2], JANUSZ MARCINKIEWICZ[1]

[1]Department of Immunology, Jagiellonian University College of Medicine, Krakow, Poland
[2]Department of Rheumatology, Jagiellonian University College of Medicine, Krakow, Poland

Abstract

Methotrexate (MTX) is used in the treatment of rheumatoid arthritis (RA) due to its anti-inflammatory properties. However, its ability to suppress macrophage derived proinflammatory cytokines has not been explained. The aim of this study was to compare the therapeutic effects of high-dose MTX on the development of collagen-induced arthritis (CIA)

High-dose MTX reduced significantly both the incidence/severity of CIA and the serum levels of IgG anti-CII. Surprisingly, macrophages obtained from MTX-treated mice and stimulated in vitro with LPS produced more TNF-α, IL-6 and IL-12p40 than the control cells. MTX added in vitro to peritoneal macrophages did not affect the cytokine production. However, incubation of proliferating RAW 264.7 macrophages with MTX resulted in severe suppression of all proinflammatory cytokines tested. In conclusion MTX treatment markedly ameliorates arthritis while it does not suppress the cytokine release from peritoneal macrophages. In vitro study indicates that MTX can inhibit the cytokine production if target cells proliferate.

Key words: *methotrexate (MTX), collagen induced arthritis (CIA), macrophages, cytokines.*

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Introduction

Methotrexate (MTX), an anti-inflammatory and anti-cancer agent, was shown to improve clinical symptoms of rheumatoid arthritis (RA) as shown for the first time in 1951 [1].

Recently, methotrexate (4-amino-N10-methylpteroyl-glutamic acid) is the most frequently used anti-rheumatic drug [2, 3]. Therapeutic effect of MTX in inflammatory rheumatic conditions and in animal models of experimental arthritis seems to be associated with its anti-inflammatory properties rather than with anti-proliferative mechanism of action. Several pharmacological mechanisms of action of MTX have been suggested so far, however, despite well documented facts there are still many controversies. There is some evidence, that MTX, a folate antagonist, may prevent *de novo* pyrimidine and purine synthesis consequently inhibiting production of DNA, RNA and proliferation of cells [4]. Most studies describe a dose-dependent induction of apoptosis and/or reduction of cell proliferation by MTX [5]. It has been suggested that MTX induces apoptosis only in activated cells [6]. Interestingly, MTX differentially affects monocytic cell lines and lymphocytes [5, 7].

In RA the rapid clinical remission after MTX application may indicate endogenous alterations in the production of cytokines [4, 8, 9] and/or humoral responses [10]. Methotrexate reduces *in vivo* the production of many

Correspondence: Janusz Marcinkiewicz, Department of Immunology, Jagiellonian University College of Medicine, Czysta 18, 31-121 Krakow, Poland, phone/fax: +48 12 633 94 31, e-mail: mmmarcin@cyf-kr.edu.pl

cytokines (TNF-α, IL-4, IL-6, IFN-γ, and GM-CSF) [4, 8, 9]. On the other hand, MTX hardly affects cytokine production by monocytes *in vitro* [8, 11].

Moreover, it has been shown that low-dose MTX-treatment of RA patients results in the reduced serum concentration of TNF-α, while *ex vivo* there is no difference in the production of TNF-α by PHA-stimulated peripheral blood mononuclear cell (PBMC) taken from RA patients and controls [12]. In addition, Aggarwal and coworkers [13] showed that 4 weeks MTX treatment has no effect on the spontaneous production of cytokines in whole-blood cultures, however after LPS stimulation IL-6 levels were lower in MTX treated patients. MTX does not affect LPS-induced release of IL-1 and TNF-α by monocyte cell lines [14]. Methotrexate also does not control *in vitro* LPS induced IL-1 synthesis by resident peritoneal macrophages [15].

As many studies on methotrexate immunosuppressive properties seem to be inconclusive and generally demonstrate little or no effect of MTX on cytokine production *in vitro*, it rises the questions whether is it a matter of:

- different target cells for MTX (macrophages vs. T cells or synoviocytes as a source of cytokines),
- expression of different receptors for MTX in distinct inflammatory loci,
- different regimes of MTX treatment used; e.g. low-dose (anti-inflammatory) vs. high-dose (anti-cancer) treatment.

Therefore, in our study we have addressed the issue whether expected beneficial therapeutic effect of high-dose MTX treatment in CIA, a murine model of RA, will be accompanied by suppression of cytokine production by any inflammatory cells, including cells not involved directly in the pathogenesis of arthritis. First of all, we wanted to explain whether the production of proinflammatory cytokines by peritoneal macrophages is resistant to MTX, both *in vitro* and *in vivo*.

Material and methods

Reagents

Collagen type II (CII) from chicken sternal cartilage, Complete Freund's Adjuvant (CFA), methotrexate (MTX), lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4, myeloperoxidase (MPO) from human leukocytes, hexadecyltrimethylammonium bromide (HTAB), o-dianisidine dihydrochloride (OPD), hydrogen peroxide were all from Sigma-Aldrich (USA). Horseradish peroxidase (HRP) conjugated streptavidin was obtained from Vector (USA) and bovine serum albumin (BSA) fraction V was from Roche Diagnostics (Germany).

Anti-mouse cytokine primary monoclonal antibodies: rat anti-mouse IL-6 monoclonal antibody, rat anti-TNF-α monoclonal antibody, rat anti-mouse IL-12p40 monoclonal antibody were all from e-Bioscience (USA). OptEIA mouse IL-10 set was from BD PharMingen (USA). Biotin conjugated antibodies: anti-IgM, anti-IgG2a were from SouthernBiotech (USA), anti-IgG1 from MP Biomedicals (USA) and anti-IgG was from Sigma-Aldrich (USA). Cytokines: recombinant mouse IL-6, recombinant mouse IL-12p40, recombinant mouse TNF-α were all from e-Bioscience (USA).

Mice

Inbred DBA/1J male mice and CBA mice were bred in the Animal Breeding Unit, Department of Immunology, Jagiellonian University College of Medicine, Krakow. Mice were housed 4-5 per cage and maintained under clean conventional conditions with free access to standard rodent diet and water. Mice were used at 8 to 10 weeks of age. The authors were granted permission by the Local Bioethical Committee to use mice in presented study. Experiments were conducted according to ethical guidance of Local Bioethical Committee.

Induction and evaluation of collagen induced arthritis

To induce collagen induced arthritis (CIA), DBA/1J mice were injected intradermally with chicken collagen type II (CII, 200 μg/mouse) emulsified in Complete Freund's Adjuvant (CFA). 21 days later mice received boost intraperitoneal injection of CII (100 μg/mouse) in the presence of LPS (5 μg/mouse). Starting from the second (booster) injection of collagen, mice were examined visually every other day for the incidence and severity of arthritis (joint swelling and redness). Paw thickness was measured using Mitutoyo micrometer. The lesions of the four paws were each graded from 0 to 4 (CIA index) according to the increasing extend of erythema and edema of the periarticular tissues.

Methotrexate treatment of collagen induced arthritis

Methotrexate (50 μg/mouse = ~2,5 mg/kg) was given by intravenous injections three times a week for 3 weeks, starting on day 21, the day of second immunization (see Protocol – Fig. 1). Control animals (referred to as untreated) received intravenous injection of saline. The regimen was chosen to test the effect of high-dose MTX on cytokine production by macrophages and was based on the protocol described by Neurath *et al.* [16].

Measurement of myeloperoxidase activity

On day 42 (the end of experiment) hind paws were collected and homogenized. Myeloperoxidase (MPO) activity in articular tissues was measured according to Bradley's method [17]. The activity of MPO was calculated from the MPO standard curve and expressed in units. One unit of MPO activity was defined as that degrading 1 μmol of hydrogen peroxide per minute at room temperature. Each sample was measured in duplicate.

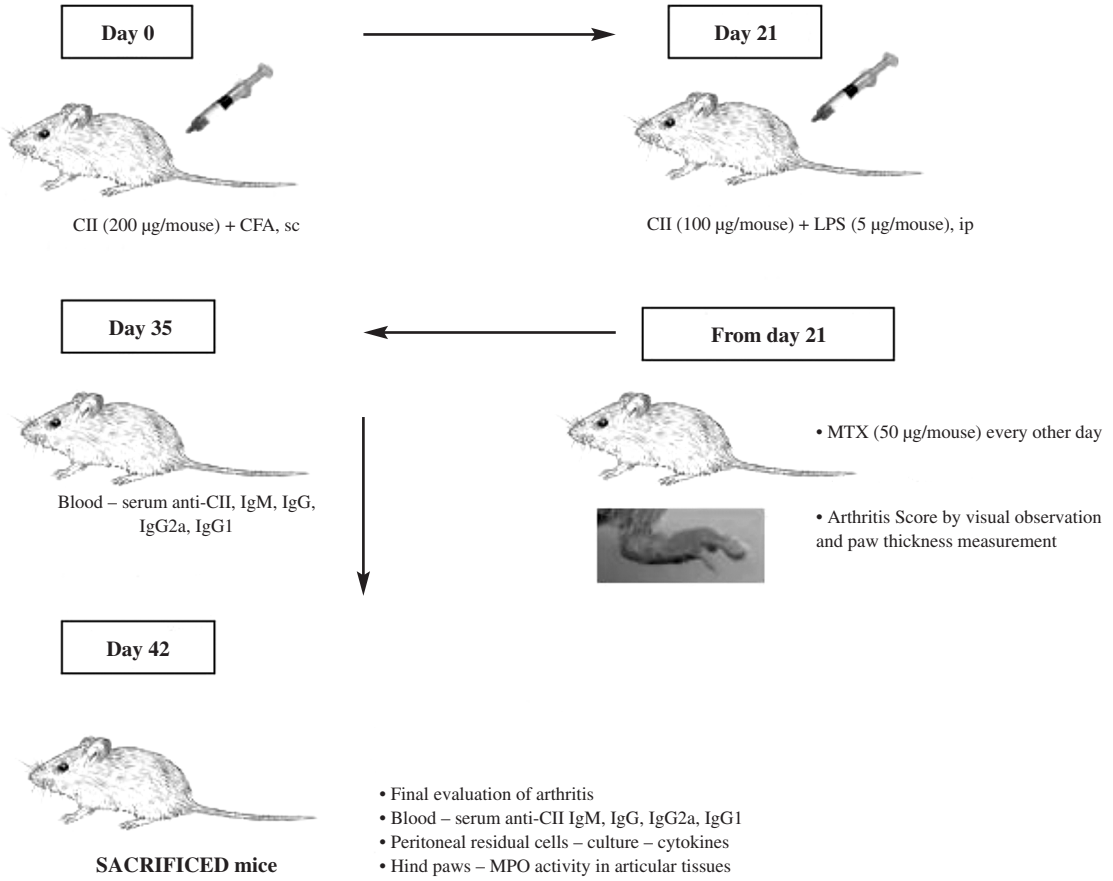

Fig. 1. Protocol of the CIA induction and MTX treatment

Mice were immunized with type II chicken collagen (CII) in CFA (200 µg/mouse, day 0) followed with the second immunization with CII (100 µg/mouse) in the presence of LPS (5 µg/mouse) 21 days later. MTX (50 µg/mouse, approx. 2.5 mg/kg) was injected intravenously (i.v.) every other day (three times a week) starting from the day of the second immunization (day 21). During treatment clinical symptoms of arthritis were visually examined three times a week and the thickness of the hind paws was measured at the same time. Two weeks after second immunization (day 35) blood was collected from all mice and the levels of anti-CII antibodies (IgM, IgG, IgG1 and IgG2a) were determined in serum. One week later (on day 42) the experiment was completed. Mice were bled and sacrificed. Residual peritoneal cavity cells were harvested for the in vitro culture and hind paws were collected for MPO assay.

Measurement of serum anti-collagen antibody titers

The blood was collected from mice on day 35 and 42 and the serum was prepared. Anti-CII antibodies in serum were measured by sandwich ELISA as described previously [18]. Shortly, plates (Costar EIA/RIA High Binding plates, Corning Incorporated, USA) were coated with native chicken collagen type II (5 µg/ml). Mouse serum diluted geometrically in PBS was applied into collagen coated wells followed with biotin-conjugated antibodies against IgM, IgG, IgG1 and IgG2a. Horseradish peroxidase conjugated streptavidin was used as detection reagent followed with the o-phenylenediamine dihydrochloride (OPD) peroxidase substrate. The antibody levels were expressed in arbitrary ELISA units calculated from anti-CII immunoglobulin titer: 1 Unit = 1/100 titer of immunoglobulin specific to native chicken collagen type II.

Measurement of cytokine production by peritoneal residual cells taken from DBA/1J mice

On day 42 (the end of experiment) peritoneal residual cells were collected and cultured at a cell density of 5×10^5/ml for 24 h with or without LPS (100 ng/ml) (*E. coli* 0111:B4 cell wall component). The concentration of cytokines (TNF-α, IL-6, IL-10, IL-12p40) in culture supernatants was measured by sandwich cytokine ELISA.

Short-term *in vivo* methotrexate treatment and its effect on *ex vivo* cytokine production by peritoneal macrophages of CBA mice

Methotrexate (50 µg/mouse) was injected intravenously on day 0, 2, 4 (7.5 mg/kg/week) to CBA mice, (referred to as MTX treated mice). Control mice were injected with saline (referred to as untreated mice). Thioglycollate was given by intraperitoneal injection on day 4 (the day of the

last injection of MTX). Three days later peritoneal exuded cells (mostly macrophages) were collected and cultured 24 h *in vitro* with or without LPS (100 ng/ml). The cytokine production was tested as described above.

In vitro effect of MTX on cytokine production by macrophages

A. Peritoneal macrophages

Naïve CBA mice were injected intraperitoneally with thioglycollate. Three days later peritoneal macrophages were collected and cultured 24 h *in vitro* with different concentrations of MTX (0, 1, 10, 100 μg/ml), with or without LPS (100 ng/ml) or cells were cultured with indicated amounts of MTX for 24 h and then medium was replaced and LPS was added to the cultured cells for additional 24 h. Cytokine production was measured in culture supernatant by ELISA.

B. RAW 264.7 – macrophage cell line

RAW 264.7 cells were cultured *in vitro* in the presence of LPS (100 ng/ml) and different concentrations of MTX (0, 1, 10, 100 μg/ml) for 24 h or cells were cultured for 24 h in the presence of MTX and then stimulated with LPS for additional 24 h. Cytokines were measured in the culture supernatant by ELISA.

Analysis of cytokine production in macrophage culture supernatant by ELISA

Cytokines in the culture supernatant were measured by sandwich cytokine ELISA.

Statistical analysis

Student's *t*-test was used to determine the significance of group differences. A *p*-value less than 0.05 was considered significant.

Results

The effect of methotrexate treatment on the development of CIA in DBA/1J mice

As expected, in mice treated with MTX (7.5 mg/kg/week) the incidence of disease and the severity of arthritis evaluated by the mean arthritis index, paw thickness and MPO activity in the articular tissues were significantly reduced (Fig. 2).

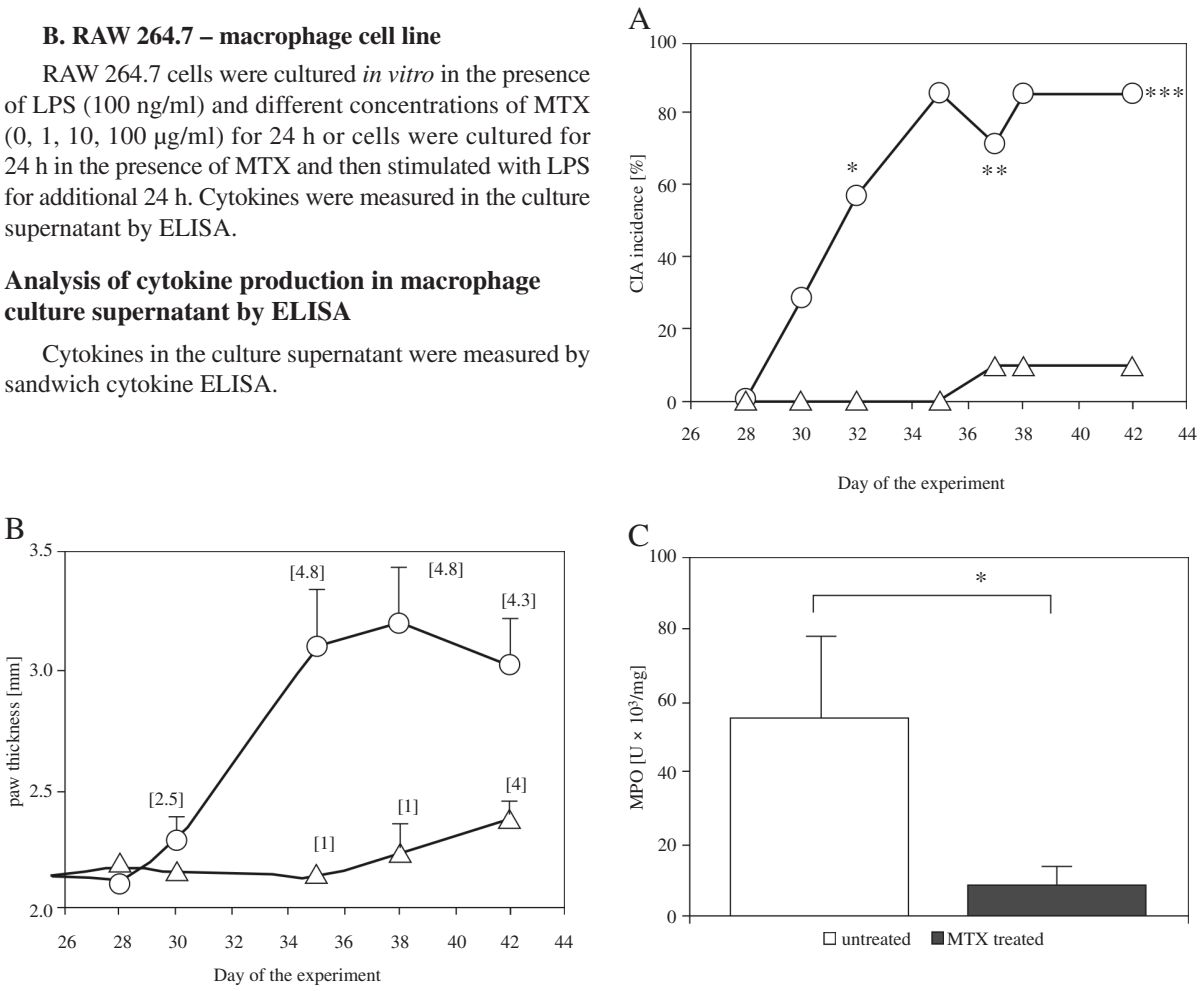

Fig. 2. The effect of MTX on the development of CIA in DBA/1J mice

A – The incidence of CIA: the percentage of the arthritic mice at the indicated times are shown.

B – The severity of arthritis is expressed as the thickness of hind paws shown in mm (mean ± SEM) and the mean of the arthritis index of all CIA positive mice is shown in parenthesis. [x] – arthritis index; ❍ – placebo (n = 8); △ – MTX (n = 10).

*C – The level of MPO measured in joint tissue of hind paws is shown as the mean of all CIA positive mice in units [U] per mg of protein obtained from hind paws. White bar – untreated; black bar – MTX treated mice. *p < 0.05, **p < 0.01; ***p < 0.001 MTX treated vs. untreated mice (Student's t-test).*

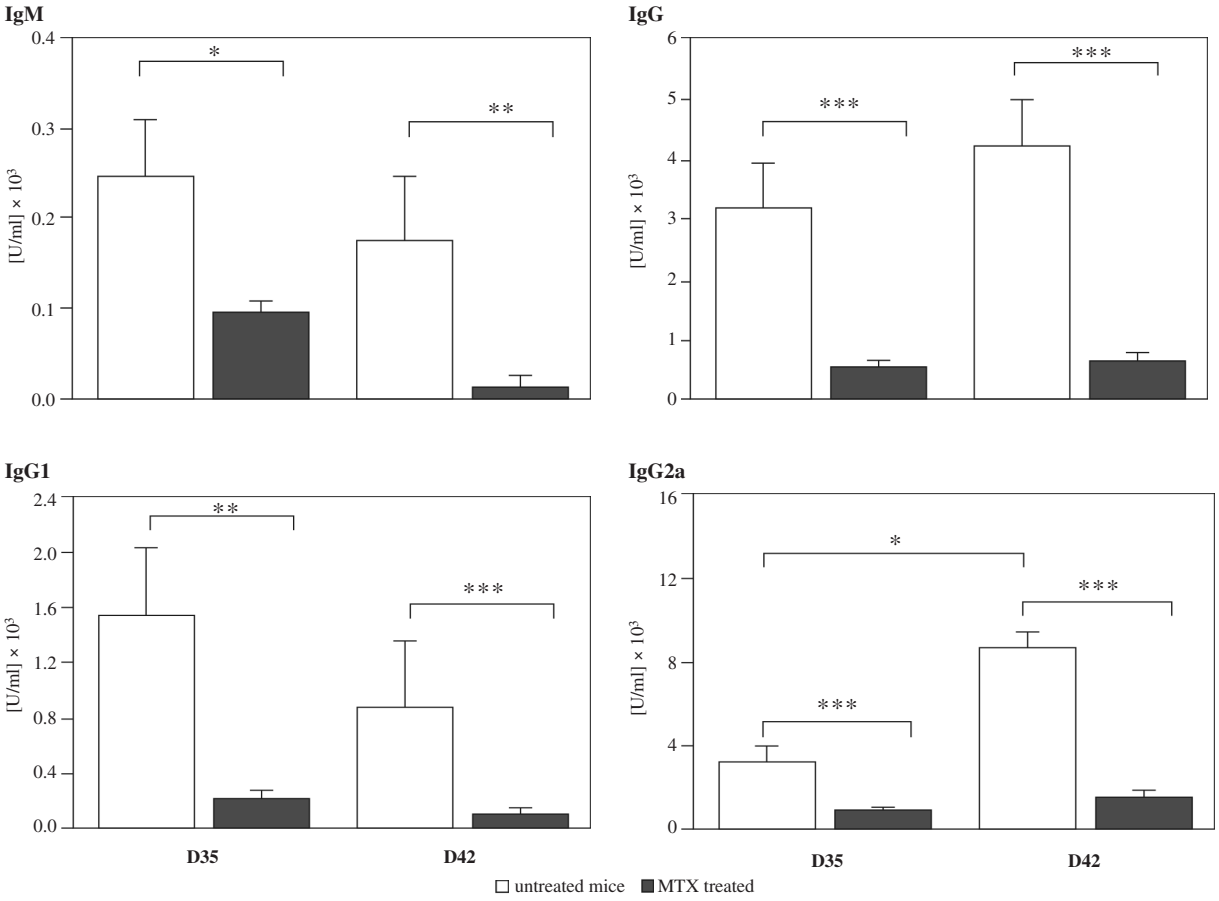

Fig. 3. The effect of MTX on CII specific antibodies in serum

*The level of anti-CII antibodies (IgM, IgG, IgG1 and IgG2a) was measured in serum one week after boost immunization, on day 35 (D35) and on day 42 (D42), at the end of the experiment. Bars show the mean ± SEM; white bars – placebo, black bars – MTX treated mice; *p < 0.05, **p < 0.01; ***p < 0.001 MTX treated vs. untreated mice (Student's t-test).*

Methotrexate treatment markedly decreased the serum levels of anti-CII antibodies (IgM, IgG, IgG2a and IgG1). The production of both anti-CII IgG2a, the indicator of Th1 response, and anti-IgG1, the indicator of Th2 response, were inhibited by high dose of MTX to the same extend (Fig. 3).

The effect of methotrexate treatment on the production of cytokines by residual peritoneal cells of DBA/1J mice

Methotrexate treatment did not affect the spontaneous *ex vivo* cytokine production by residual peritoneal cells of DBA/1J mice (data not shown). Surprisingly, cells taken from MTX-treated mice and stimulated *in vitro* with LPS, showed higher production of proinflammatory cytokines (TNF-α, IL-6 and IL-12p40) as compared to the peritoneal cells of untreated mice, while IL-10 production was not affected by MTX-treatment (Fig. 4).

The effect of short time *in vivo* methotrexate treatment on the cytokine production by thioglycollate-induced peritoneal macrophages of CBA mice

Short term *in vivo* MTX treatment did not affect the spontaneous *ex vivo* cytokine production by thioglycollate-induced peritoneal macrophages. However, in response to *in vitro* stimulation with LPS of cells taken from mice treated with MTX *in vivo* (3 intravenous injections of MTX, 50 μg/mouse) produced more proinflammatory cytokines (IL-6, TNF-α and IL-12p40) than cells taken from untreated mice (saline injected mice) (Fig. 5).

In vitro effect of methotrexate on cytokine production by thioglycollate-induced CBA peritoneal macrophages

When thioglycollate-induced peritoneal macrophages from naïve CBA mice were cultured *in vitro* with LPS and

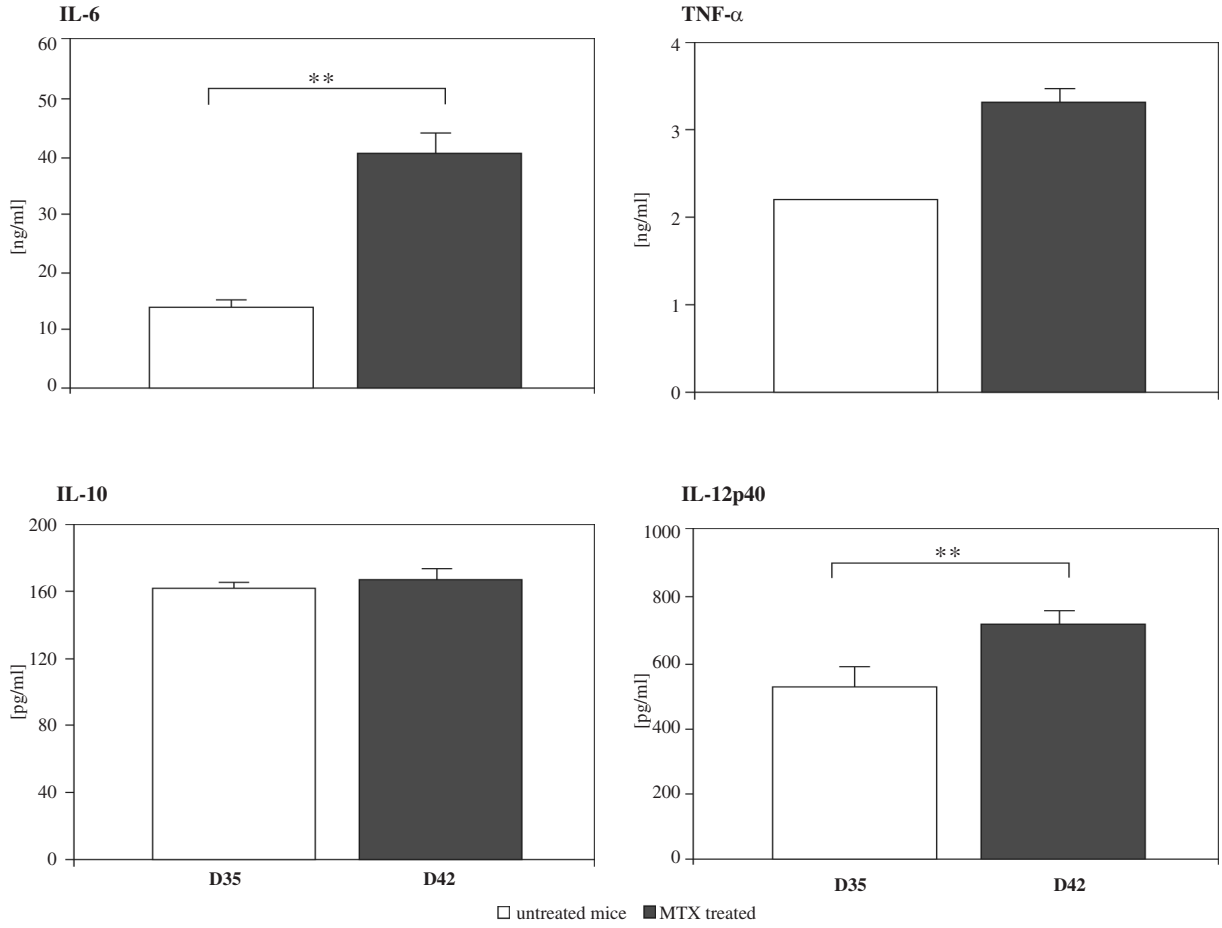

Fig. 4. The effect of MTX on the production of cytokines by peritoneal cells of DBA/1J mice

*Residual peritoneal cells of MTX treated and untreated DBA/1J mice were cultured in vitro for 24 h in the presence of LPS (100 ng/ml). IL-6, TNF-α, IL-10 and IL-12p40 in the culture supernatants were measured by ELISA. White bars – untreated, black bars – MTX treated mice; **p < 0.01; MTX treated vs. untreated mice (Student's t-test).*

different concentrations of MTX (1-100 μg/ml), MTX had no effect on LPS-induced cytokine production by these cells (Fig. 6A). However, when the thioglycollate-induced peritoneal macrophages were pretreated with MTX *in vitro* for 24 h and then stimulated with LPS, they produced more proinflammatory cytokines (IL-6, TNF-α and IL-12p40). The level of IL-10 was not affected by MTX treatment *in vitro* (Fig. 6B).

In vitro effect of methotrexate on cytokine production by RAW 246.7 macrophage cell line

In the culture of RAW 264.7 cells stimulated *in vitro* with LPS MTX at low concentration (1 or 10 μg/ml) did not affect the production of proinflammatory cytokines (IL-6 and TNF-α) by that macrophage cell line. However, in the presence of high concentration of MTX (100 μg/ml) LPS stimulated RAW 264.7 cells produced less TNF-α and IL-6 as compared to MTX-untreated cells. The level of IL-10 production by RAW 264.7 cells was decreased by MTX in a dose dependent manner (Fig. 7A). When the RAW 264.7 cells were treated with MTX (24 h culture *in vitro*) prior to stimulation with LPS they produced significantly less cytokines tested (IL-6, TNF-α and IL-10; IL-12p40 was not detectable) (Fig. 7B).

Discussion

Methotrexate is the most commonly used slow acting immunosuppressive drug for RA and other autoimmune diseases. In contrast to anticancer treatment (e.g. in acute lymphoblastic leukemia), very low doses of MTX are given weekly in RA (15-30 mg/week ≤ 0.3 mg/kg), as a standard procedure [2]. However, high-dose MTX has been also used in arthritis in the treatment of refractory juvenile rheumatoid

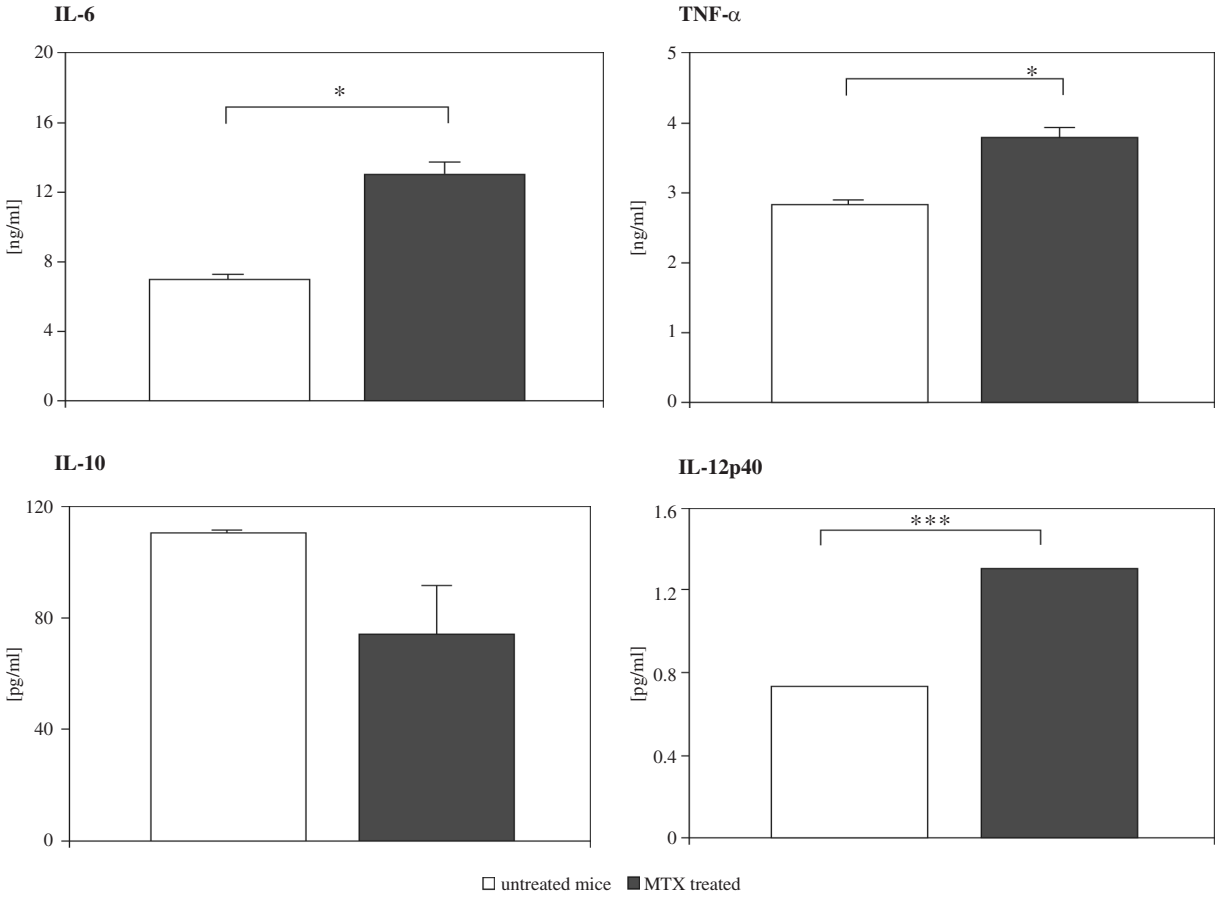

Fig. 5. The effect of short time *in vivo* MTX treatment on the cytokine production by CBA mice peritoneal macrophages

*CBA-mice received 3 intravenous injections of MTX (50 µg/mouse) every other day. Then thioglycollate was injected intraperitoneally to induce macrophages. Peritoneal macrophages were harvested three days later and the cells were cultured in vitro for 24 h in the presence of LPS (100 ng/ml). IL-6, TNF-α, IL-10 and IL-12p40 were measured in the culture supernatants by ELISA. White bars – untreated, black bars – MTX treated mice; *p < 0.05; ***p < 0.001, MTX treated vs. untreated mice (Student's t-test).*

arthritis [19]. The beneficial therapeutic effect of MTX in RA has been shown to be associated with anti-inflammatory properties of the drug, especially with the inhibition of proinflammatory cytokines such as TNF-α [8].

Collagen-induced arthritis (CIA) in mice is a commonly used animal experimental model for studying arthritis, the most relevant model to RA in humans. Collagen-induced arthritis has been also used to examine the immunosuppresive and anti-inflammatory properties of MTX. The effects of MTX treatment on both clinical symptoms and *ex vivo* activities of immune cells (macrophages, T cells, synoviocytes, epithelial cells) has been studied in many laboratories. It is a common agreement that MTX ameliorates the development CIA and is accompanied with inhibition of the production of proinflammatory mediators (cytokines, eicosanoids, NO). However, the major target cell for MTX, the producer of cytokines, is still controversial. Especially the data showing the effect of MTX on macrophage activity *ex vivo* and *in vitro* are not consistent. It has been shown that MTX does not affect, or only slightly inhibits, the cytokine production by macrophages, while it strongly inhibits the production of T-cell cytokines [4, 15]. On the other hand, low-doses of MTX significantly diminished the production of TNF-α, IL-1 and IL-6 by human synovial macrophages [20]. The differences between *in vivo* effects of MTX on human (RA) and murine (CIA) macrophages may be explained by treatment of mice with the doses of MTX not relevant to those used for treatment of RA patients.

In this study we have tested the MTX effect on cytokine production by macrophages in three experimental systems:

- MTX treatment of CIA in DBA/1J mice and *ex vivo* cytokine release by residual peritoneal cells,
- short-term *in vivo* MTX treatment of CBA mice and *in vitro* examination of thioglycollate-induced peritoneal macrophages,
- *in vitro* incubation of MTX with either CBA mice peritoneal macrophages or with RAW 264.7 cells (murine macrophage cell line).

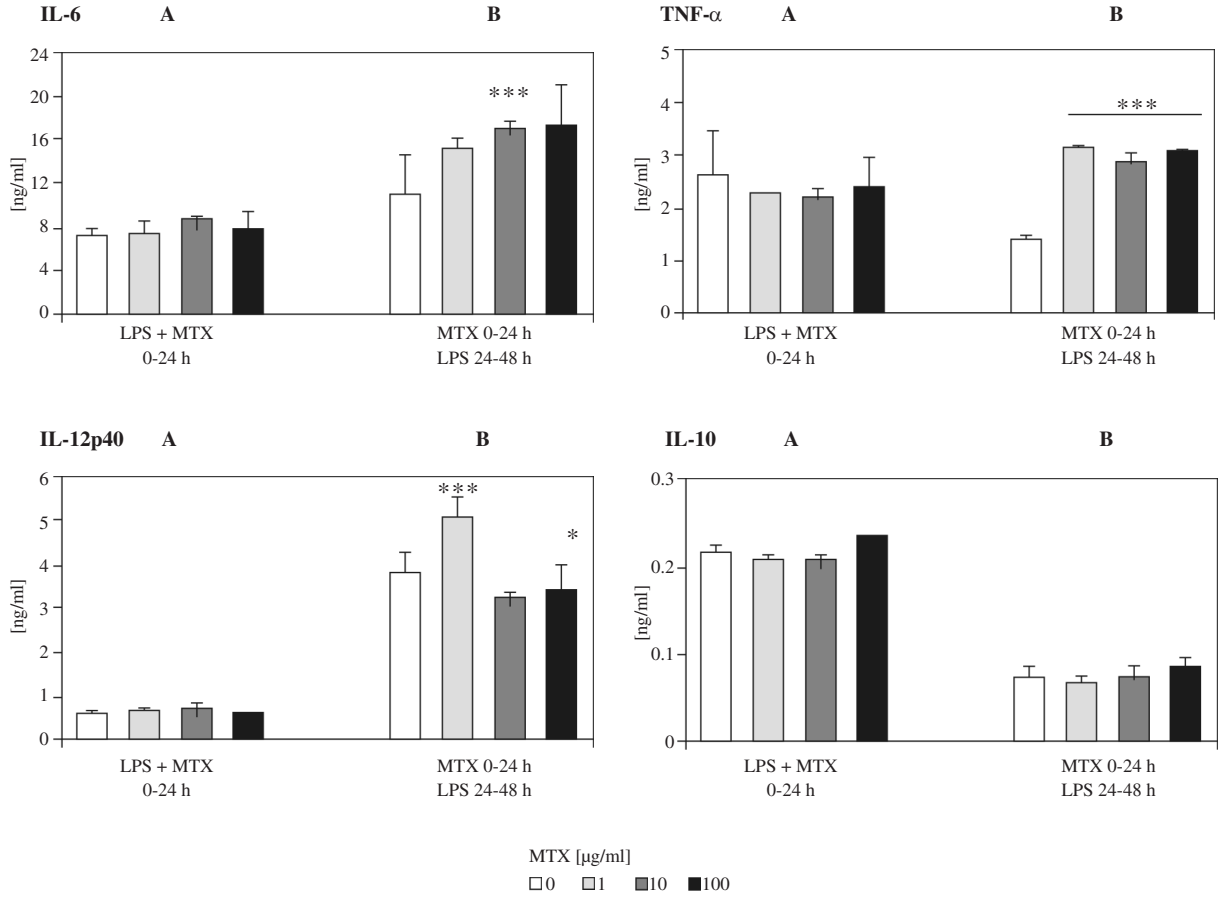

Fig. 6. *In vitro* effect of MTX on cytokine production by thioglycollate-induced CBA peritoneal macrophages

*A – Peritoneal macrophages were cultured in vitro in the presence of LPS (100 ng/ml) and various concentrations of MTX (0 – white bars, 1, 10 or 100 μg/ml – gray to black bars) for 24 h or B – macrophages were cultured for 24 h in the presence of MTX and then stimulated with LPS for additional 24 h. IL-6, TNF-α, IL-12p40 and IL-10 were measured in the culture supernatant by ELISA; *p < 0.05; ***p < 0.001, MTX treated vs. untreated cells (Student's t-test).*

We have used high-dose MTX treatment *in vivo* (7.5 mg MTX/kg/week) and *in vitro* (1, 10, 100 μg of MTX/ml), according to the protocols used previously by Neurath, to test MTX specific effects on proinflammatory cytokine production in healthy and arthritic (CIA) mice [16].

Our studies clearly indicate that intravenous MTX administration markedly ameliorates arthritis in CIA-induced mice. The attenuation of clinical symptoms was associated with the profound inhibition of anti-collagen II immunoglobulin production. This therapeutic effect was similar to that observed by Neurath *et al.* after intraperitoneal administration of MTX [16]. There was no difference between the effect of MTX on Th1 and Th2 related IgG subclasses, IgG2a and IgG1, respectively, in contrast to other studies, in which MTX selectively inhibited Th1 response [21, 22]. It has been found that MTX suppresses production of proinflammatory cytokines such as IL-6, IL-1, TNF-α and IFN-γ *in vitro*, *ex vivo* and *in vivo*, while production of the anti-inflammatory Th2 IL-4 cytokine was less affected [11]. Moreover, the production of T-cell cytokines (TNF-α) was more affected than the production of TNF-α by macrophages. It was the effect of MTX on spleen cells taken from arthritic mice and activated *in vitro* with either T-cell (anti-CD3/CD28 antibodies) or macrophage (LPS – TLR4 ligand) stimuli [16, 23]. These data suggest that TNF-α production by T cells is an important target of MTX. In the present study we addressed the question whether macrophages are the target cells for MTX in arthritis. Surprisingly, we have found that MTX treatment, starting prior to the CIA onset, resulted in the enhancement of cytokine production (TNF-α, IL-6, IL-12) by peritoneal cells stimulated *in vitro* with LPS, as compared to cytokine production by cells taken from untreated DBA/1J mice. This effect was also observed when thioglycollate-induced peritoneal macrophages were taken from CBA mice treated with MTX and compared with control macrophages. Interestingly, the production of proinflammatory cytokines (TNF-α, IL-6 and IL-12) was more affected than that of anti-inflammatory IL-10. The effect was not limited to LPS stimulation because the same

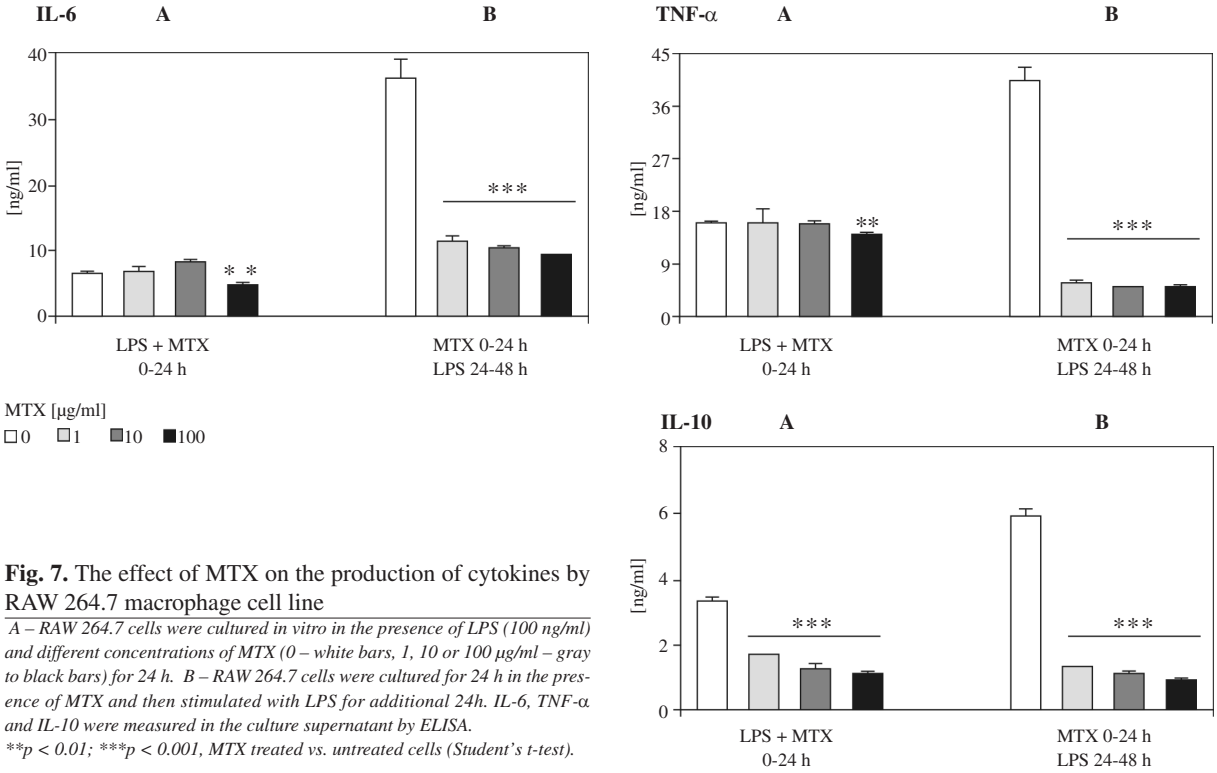

Fig. 7. The effect of MTX on the production of cytokines by RAW 264.7 macrophage cell line

A – RAW 264.7 cells were cultured in vitro in the presence of LPS (100 ng/ml) and different concentrations of MTX (0 – white bars, 1, 10 or 100 µg/ml – gray to black bars) for 24 h. B – RAW 264.7 cells were cultured for 24 h in the presence of MTX and then stimulated with LPS for additional 24h. IL-6, TNF-α and IL-10 were measured in the culture supernatant by ELISA.
***p < 0.01; ***p < 0.001, MTX treated vs. untreated cells (Student's t-test).*

results were seen after the stimulation of macrophages with zymosan (data not shown). These data suggest indirect *in vivo* effect of MTX on macrophages. One may speculate that *in vivo* MTX selectively inhibited some undetected macrophage suppressor(s), what resulted in an enhancement of the production of proinflammatory mediators such as TNF-α, IL-6 and NO (not shown). On the other hand, there exists a great body of evidence that MTX directly inhibits proinflammatory functions of macrophages. It has been shown that suppression of inflammation (thioglycollate-induced peritonitis) by low-dose MTX is mediated by activation of adenosine A_{2A} receptor in peritoneal cells and by reduced leukocyte accumulation at a site of inflammation. However, it has been also postulated that the anti-inflammatory effects of MTX are mediated by different receptors in different inflammatory loci or on different inflammatory cells [24]. The latter hypothesis may explain the observed dual effect of MTX treatment of CIA, the anti-inflammatory effect on the development of arthritis and the "proinflammatory" effect on peritoneal cells in DBA/1J mice. To investigate the direct effect of high-dose MTX on cytokine production by macrophages we extended our studies on *in vitro* experimental model. Interestingly, MTX (1-100 µg/ml), added to CBA mice peritoneal macrophages simultaneously with LPS, did not inhibit the production of proinflammatory cytokines and in some experiments it even slightly enhanced this production. Finally, we have tested *in vitro* the influence of MTX on RAW macrophages. We have used macrophage cell line for two reasons. First, we wanted to avoid the presence of additional inflammatory cells, which could be the primarily target of MTX. Second, in contrast to peritoneal macrophages, RAW macrophages are proliferating cells. Therefore, MTX may halt proliferation of these dividing cells at G1 phase of the cell cycle, similarly to its action on macrophage synoviocytes in arthritic joints. We have found that MTX markedly suppresses production of proinflammatory cytokines when pre-incubated with proliferating RAW cells for 24 h before their stimulation with either LPS or zymosan (data not shown).

In conclusion, the data reported here provide evidence that high-dose MTX-treatment markedly ameliorates arthritis while it does not suppress the cytokine release from peritoneal macrophages. It may suggest different biological targets for MTX (e.g. synoviocytes vs. peritoneal macrophages). Moreover, the *in vitro* study clearly indicates that MTX can inhibit the cytokine production if target cells proliferate. However, further studies are necessary to explain the mechanism of MTX-dependent sensitization of peritoneal macrophages to enhanced production of proinflammatory mediators.

Acknowledgments

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